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# Modifications to the Hemagglutinin Cleavage Site Control the Virulence of a Neurotropic H1N1 Influenza Virus<sup>∇</sup>

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A key determinant of influenza virus pathogenesis is mutation in the proteolytic cleavage site of the hemagglutinin (HA). Typically, low-pathogenicity forms of influenza virus are cleaved by trypsin-like proteases, whereas highly pathogenic forms are cleaved by different proteases (e.g., furin). Influenza virus A/WSN/33 (WSN) is a well-studied H1N1 strain that is trypsin independent in vitro and has the ability to replicate in mouse brain. Previous studies have indicated that mutations in the neuraminidase (NA) gene allow the recruitment of an alternate protease (plasminogen/plasmin) for HA activation. In this study we have identified an additional mutation in the P2 position of the WSN HA cleavage site (S328Y) that appears to control virus spread in a plasmin-dependent manner. We reconstructed recombinant WSN viruses containing tyrosine (Y), phenylalanine (F), or serine (S) in the P2 position of the cleavage site. The Y328 and F328 viruses allowed plaque formation in the absence of trypsin, whereas the S328 virus was unable to form plaques under these conditions. In mice, Y328 and F328 viruses were able to efficiently spread following intracranial inoculation; in contrast, the S328 virus showed only limited infection of mouse brain. Following intranasal inoculation, all viruses could replicate efficiently, but with Y328 and F328 viruses showing a limited growth defect. We also show that wild-type HA (Y328) was more efficiently cleaved by plasmin than S328 HA. Our studies form the foundation for a more complete understanding of the molecular determinants of influenza virus pathogenesis and the role of the plasminogen/plasmin system in activating HA.

For all viruses, the infectious cycle begins with the penetration of the host cell (27). Enveloped viruses penetrate cells via a membrane fusion event mediated by a spike protein present in the virus envelope, with fusion triggered by conformational changes in the spike protein following exposure to low pH and/or receptor (45). In the case of influenza virus, the viral spike protein hemagglutinin (HA) mediates both receptor binding and membrane fusion (46). Many viral fusion proteins are activated following cleavage by host cell proteases (9, 20, 21), and this has been most extensively documented for influenza viruses, where cleavage is directly related to exposure of the fusion peptide and fusion activation (38). For proteases, a general nomenclature for the cleavage site positions of the substrate has been designated, with cleavage occurring between P1 and P1' and with the position numbers increasing in the N-terminal direction relative to the cleaved peptide bond (P2, P3, P4, etc.). Low-pathogenicity influenza virus strains contain an HA cleavage site with a single arginine residue at the P1 position and are thus described as having monobasic cleavage sites. These viruses can utilize trypsin (or other trypsin-like serine proteases) for activation, with the tissue distribution of the activating protease typically restricting infection to the respiratory and/or intestinal organs. The presence of a

polybasic cleavage site is critical for the systemic spread and increased virulence associated with highly pathogenic avian influenza (HPAI) viruses (33). In the case of HPAI viruses such as H5N1 and H7N7, it is well established that mutations in the region of the HA cleavage site lead to an insertion of several arginine or lysine residues in addition to the P1 arginine (specifically in the P2 to P6 cleavage site positions) that can be recognized by furin—an intracellular serine protease found in many cell types—allowing a widening of the cell tropism of the virus (18).

Influenza virus is currently of major biomedical interest, both due to annual morbidity and the threat of new pandemic viruses. Influenza viruses exist as many different subtypes (H1 to H16), with H1 and H3 viruses currently infecting humans (10, 30). Normally, H1 viruses are considered to have low pathogenicity and have a monobasic cleavage site. However, two H1 isolates A/WSN/33 (WSN) and A/NWS/33 (NWS) have been selected to propagate in mouse brain and are thus considered to be highly pathogenic, neurovirulent viruses in mice (39, 40). The A/WSN/33 virus in particular has been used extensively for studies on influenza virus replication and pathogenesis, in part because this virus forms plaques in the absence of trypsin and serves as a model of highly pathogenic influenza virus. The HA of the A/WSN/33 virus was originally shown to be cleaved by plasmin, following activation of serum plasminogen in MDBK cells (22). The virulence properties of A/WSN/33 were subsequently linked to the neuraminidase (NA) gene (37) and the absence of a glycosylation site at position 130 of NA (25). In addition, the presence of a C-terminal lysine on NA was shown to be critical for the virulence properties of A/WSN/33, with the viral NA binding and sequestering of plasminogen on the cell surface,

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leading to increased cleavage of HA (15, 16). It has also been suggested that the HA of A/WSN/33 can be cleaved by an endosomal serine protease in MDBK cells (7).

Based on the recent pandemic status of novel H1N1 viruses and the known importance of the HA cleavage site in viral pathogenicity (30), we assessed the presence of cleavage site changes, in addition to the conventional monobasic/polybasic cleavage sites, in the context of the virulence properties of H1 influenza viruses. We characterize the role of the bulky hydrophobic residues tyrosine and phenylalanine, found in the P2 cleavage positions of only A/WSN/33 and A/NWS/33 HA, respectively, and show that these residues are major virulence determinants for these viruses, allowing efficient use of plasmin for spread *in vitro* and *in vivo*.

### MATERIALS AND METHODS

Cells, viruses, and plasmids. 293 T cells and CV-1 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Cellgro) with 10% fetal bovine serum (Gibco), 100 units/ml penicillin (Cellgro), and 10 µg/ml streptomycin (Cellgro). MDBK cells were maintained in Eagle's modified essential medium (EMEM) (Cellgro) with 10% fetal bovine serum, 100 units/ml penicillin, and 10 µg/ml streptomycin. The HA gene was amplified from A/WSN/33-infected MDBK cells. Viral RNA (vRNA) was extracted using an RNeasy Kit (Invitrogen) and then used as the template for reverse transcription-PCR (RT-PCR) with HA universal primers using a One Step RT-PCR kit (Qiagen). The RT-PCR products were first cloned into pCR2.1-TOPO vector (Invitrogen) to verify sequences and subsequently cloned into pEF4/myc-His vector (Invitrogen) for protein expression. The HA sequence of pEF4-WSN-HA constructed from our laboratory A/WSN/33 virus has three amino acid modifications (at amino acids 64, 390, and 398) compared to the HA from the plasmid pPOL1-WSN-HA, which was used for reverse genetics experiments. Site-directed mutagenesis was performed using a Quick Change sitedirected mutagenesis kit from Stratagene (La Jolla, CA) to introduce mutations into pEF4-WSN-HA and pPOL1-WSN-HA. According to the manufacturer's protocol, pairs of complementary oligonucleotides were designed to introduce the desired mutations. The sequences of the HA gene for the wild-type (Y328) and mutant (Y328F and Y328S) forms of HA were confirmed by sequencing using an Applied Biosystems Automated 3730 DNA Analyzer at the Cornell University Life Sciences Core Laboratories Center.

**Proteases.** Trypsin [L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated; from bovine pancreas] was obtained from Pierce. Plasmin was obtained from EMD Biosciences. The plasmin inhibitor 6-amino-*n*-hexanoic acid (AHA) was obtained from Alfa Aesar.

Plaque assay and viral multicycle growth assay. For plaque assays, confluent MDBK cells grown in 12-well plates were washed with phosphate-buffered saline (PBS) and then incubated with 10-fold serially diluted virus with RPMI 1640 medium (Cellgro) containing 0.2% bovine serum albumin ([BSA] essentially protease free; Sigma) buffered to pH 6.8 with HEPES (BioWhittaker) for 1 h at 37°C. The viral inocula were removed, and the infected cells were washed again with PBS before the addition of EMEM containing 0.2% BSA and 1% Sea-Plaque agarose (Lonza). TPCK-trypsin (0.5  $\mu$ g/ml) was added to the agarose overlay medium when required. The plaques were counted at 48 h postinfection after cells were fixed with 10% formalin for 20 min and stained with 0.1% crystal violet for 10 min.

In the viral multicycle growth assay, MDBK cells were incubated with influenza virus at a multiplicity of infection (MOI) of 0.01 or 0.001, diluted in RPMI 1640 medium containing 0.2% BSA, pH 6.8, for 1 h at 37°C. The viral inocula were then removed, and cells were washed with PBS and incubated with EMEM containing 0.2% BSA. TPCK-trypsin was used at a concentration of 0.5 mg/ml, and the plasminogen inhibitor AHA was used at a concentration of 5 mg/ml. The supernatants from infected cells were harvested every 12 h until 72 h postinfection, and the viral titers of the harvested supernatants were determined by plaque assay in MDBK cells with TPCK-trypsin (0.5  $\mu g/ml$ ).

Biotinylation of surface proteins and immunoprecipitation. MDBK cells were infected for 8 h, and CV-1 cells were infected for 16 h before cells were processed for surface protein biotinylation in order to analyze the cleavage of cell surface-expressed HA. Cells were grown in 12-well plates, prewashed twice with ice-cold PBS, and then labeled with 250  $\mu$ g/ml sulfosuccinimidyl 2-[biotinamido]ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin; Pierce) for 30 min on ice. Unlabeled

free biotin was quenched by addition of 150 mm of ice-cold glycine in PBS (pH 7.4) for 20 min. The cells were then lysed in 500  $\mu l$  of radioimmunoprecipitation assay (RIPA) buffer (100 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid, pH 7.4) containing complete protease inhibitor mixture (Roche Applied Science). The surface-expressed HA protein was concentrated and purified by NeutrAvidin agarose resin (Pierce) immunoprecipitation and then analyzed by Western blotting using goat anti-influenza virus A/PR8 HA antibody (NIAID Biodefense and Emerging Infections Research Resource Repository).

Plasmin-mediated HA cleavage assay. Cells were grown in 12-well plates and transfected with 500 ng of wild-type- or mutant HA-expressing plasmids using ExGen 500 for 24 h at 37°C. The transfected cells were washed with PBS twice and then were treated with 105 mU/ml plasmin (Calbiochem) diluted in DMEM at 37°C. In order to analyze plasmin-mediated HA cleavage, the cells were processed for surface protein biotinylation, followed by Western blot analysis using goat anti-A/PR8 HA antibody. To analyze HA cleavage of released virions from infected MDBK cells, the supernatants of the infected MDBK cells were collected at 12 h postinfection before Western blot analysis. Images of Western blots were obtained using an LAS-3000 MiniFujifilm imaging system (Fuji Photo Film Co., Ltd), and pixel intensity was measured for the HA0 (precursor protein) and HA1 (the cleaved product of HA0) bands. HA cleavage efficiency was determined using the following formula: HA1/(HA1 + HA0)  $\times$  100.

Generation of mutant influenza viruses by reverse genetics. For generation of recombinant influenza viruses, 12 plasmids (pCDNA3-PB1, pCDNA-PB2, pCDNA-PA, pCDNA-NP, and 8 plasmids encoding the individual viral RNA segments) for influenza virus A/WSN/33 were kindly provided by George Brownlee, Oxford University, United Kingdom, and recombinant viruses were generated according to procedures described previously(12). Briefly, 293 T cells from a six-well plate were transfected with eight pPOLI plasmids expressing vRNAs along with four plasmids expressing NP, PB1, PB2, and PA protein for 48 h using ExGen 500 (Fermentas). The collected transfected cell supernatants were treated with TPCK-trypsin at 1 μg/ml for 15 min at 37°C before being added to MDBK cells for plaque purification. The plaque-purified recombinant viruses were then passaged three times in 11-day-old embryonated chicken eggs, and the HA sequences of the rescued recombinant viruses were confirmed to have no extra mutations except at the P2 cleavage site position.

Infection of influenza virus in mice. To determine viral pathogenicity in mice, eight 4-week-old female BALB/c mice for each group were anesthetized by isoflurane inhalation and then injected intracranially with 10<sup>3</sup> PFU of virus in 100  $\mu$ l of PBS or inoculated intranasally with 2  $\times$  10<sup>5</sup> PFU of virus diluted in 50  $\mu$ l of PBS. Brain tissue from intracerebral infection groups and lung tissue from intranasal infection groups were removed at 3 days postinfection after mice were euthanized. The collected tissues were weighed, and 10% homogenates were prepared in cold PBS. The homogenates were centrifuged at 3,000 rpm for 10 min to remove cell debris, and then the supernatants were 10-fold serially diluted for viral titer determination by plaque assay in MDBK cells with 0.5  $\mu g/ml$ trypsin. Data were analyzed by SPSS software (version 15.0). All P values were determined by one-way analysis of variance (ANOVA). P values of <0.05 were considered to be statistically significant. All work with animals was carried out according to the Cornell University Animal Care and Use program under Animal Welfare Assurance A3347-01 and complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

## RESULTS

Bioinformatic analysis of the influenza virus H1 cleavage site. A multiple sequence alignment of the HA gene of representative H1 influenza viruses reveals an invariant arginine at position 329 (numbering based on H3 subtype virus A/Aichi/2/68), which comprises the P1 position of the proteolytic cleavage site (Fig. 1). In addition to the invariant arginine residue at the cleavage site, H1 influenza viruses contain a highly conserved serine residue at position 328 (the P2 cleavage position). The only two exceptions to this are A/WSN/33 and A/NWS/33, which contain tyrosine and phenylalanine, respectively, at position 328.

We extended our survey of the HA P2 cleavage position to other HA subtypes. While the P1 position is essentially invariant, the P2 position does show variability in the amino acid

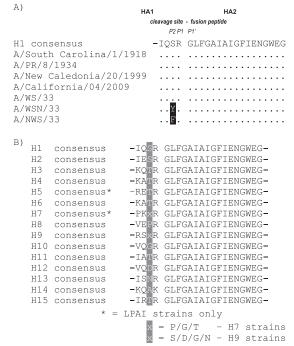


FIG. 1. Multiple sequence alignment of HA in the vicinity of the proteolytic cleavage site. (A) Sequence alignment of H1 HA. A total of 3,425 H1 HA sequences from the NCBI Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) were aligned using Clustal W, and the aligned sequences of representative viruses at the HA cleavage site are shown, along with the consensus sequence. Dots indicate identical residues, and the P2-P1' positions of the cleavage site and the location of the fusion peptide are shown. The representative viruses shown are the following: A/South Carolina/1/1918 (accession number AAD17229), A/PR/8/1934 (ABO21709), A/New Caledonia/20/1999 (ABF21272), A/California/04/2009 (ACP41105), A/WS/33 (ABD77796), A/WSN/33 (ABF47955), and A/NWS/33 (AAA92279). (B) Sequence alignment of H1 to H15 HAs. All HA sequences were obtained from the NCBI Influenza Virus Resource, and each HA subtype was aligned individually using Clustal W. For H5 and H7 HA alignments, sequences containing a polybasic cleavage site were not included. The consensus sequence of each subtype is shown. LPAI, low-pathogenic avian influenza virus.

residue. Within an HA subtype there is usually a predominant P2 residue, with the exception of the H7 and H9 viruses, where there are several dominant P2 residues. However, in all cases, the P2 cleavage site contains a residue with an uncharged, aliphatic or charged R group and not an aromatic R group, as in the case of the neurotropic H1 strains.

The reverse-genetics-engineered influenza WSN HA mutant Y328S virus fails to undergo multicycle replication in MDBK cells in the absence of trypsin. To determine whether the tyrosine residue at the P2 position (Y328) of the wild-type WSN HA cleavage site has any effect on HA precursor protein cleavage and virus activation, we generated recombinant wildtype viruses, along with mutant viruses containing a serine or phenylalanine mutation at HA residue 328, using reverse genetics. The serine mutant virus (HA Y328S) was generated to mimic the conserved sequence at the HA cleavage site P2 position, and the phenylalanine mutant virus (HA Y328F) was generated to mimic the sequence present in the A/NWS/33 virus. Recombinant wild-type, Y328S, and Y328F viruses could be rescued from transfected 293 T cells and subsequently amplified in MDBK cells with the addition of trypsin. According to previous reports, WSN can undergo multicycle replication in MDBK cells without exogenous trypsin. We therefore examined whether the mutant Y328S and Y328F viruses retain the ability of trypsin-independent replication in MDBK cells. In plaque assays in MDBK cells, we found that the titers of the WSN wild type (Y328) or the Y328F mutant virus were comparable with or without exogenous trypsin. In contrast, only the lower dilutions of the Y328S mutant were able to form plaques in the absence of trypsin, resulting in a significant reduction in virus titer (Fig. 2A). The growth defect of the Y328S mutant virus in MDBK cells in the absence of trypsin was then confirmed in viral multicycle growth experiments, in which MDBK cells were infected with either wild-type (Y328) or mutant Y328S and Y328F viruses at an MOI of 0.01 for 72 h, with the supernatants being harvested every 12 h for viral titer determination. In the presence of trypsin, both Y328S and Y328F mutant viruses replicated as efficiently as the WSN wild type and could reach a titer of  $10^8\,\mathrm{PFU}$  per ml by 72 h postinfection (Fig. 2B). When trypsin was not included in the culture me-

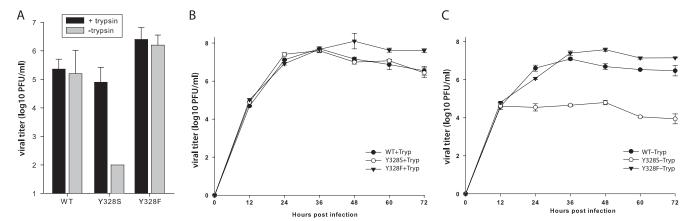


FIG. 2. Growth properties of recombinant wild-type and mutant WSN viruses. (A) Viral plaque assay in MDBK cells with or without trypsin (0.5  $\mu$ g/ml). (B and C) MDBK cells were infected with WSN wild-type (WT) or mutant WSN HA Y328S or Y328F virus at an MOI of 0.01 for up to 72 h with (B) or without (C) trypsin. The supernatants from infected cells were harvested every 12 h for plaque assay in MDBK cells with trypsin (0.5  $\mu$ g/ml). Error bars represent the standard error of the mean for three independent experiments.

dium, the WSN wild-type (Y328) and Y328F mutant viruses did not show any defects in viral multicycle replication, and the virus titers were comparable to those obtained in the presence of trypsin. However, while Y328S mutant virus replicated similarly to the wild type by 12 h postinfection, the virus titer did not increase after this time, and the titer was approximately 2 log units lower than that of the wild type at 72 h, suggesting that the released viruses from the first-round infection were not infectious (Fig. 2C). Overall, these data provide strong evidence that a tyrosine (or other bulky aromatic) residue at the P2 position of the HA cleavage site is important for the trypsin-independent multicycle replication of the WSN virus in MDBK cells, with a serine residue at this position greatly reducing the ability of the virus to undergo multicycle replication.

The serine protease plasmin/plasminogen is responsible for WSN HA cleavage in MDBK cells. Based on the fact that the broad-spectrum serine protease inhibitor aprotinin could efficiently block WSN virus trypsin-independent replication (7), it has been previously suggested that a serine protease is responsible for WSN HA cleavage in MDBK cells. We confirmed these data and next investigated which specific serine protease might be responsible for WSN HA cleavage in MDBK cells. The lysine analog 6-amino-n-hexanoic acid (AHA), a competitive inhibitor of serine protease plasminogen and plasmin, efficiently inhibited WSN virus plaque formation in MDBK cells (data not shown). The inhibitory effect of AHA on WSN virus multicycle replication in MDBK cells was further verified in viral multicycle growth assays, in which MDBK cells were infected with WSN virus at an MOI of 0.001 in the presence of AHA (5 mg/ml). In contrast to the typical high virus titer of up to 10<sup>8</sup> PFU per ml by 72 h postinfection, the WSN virus titer was lower than our detection level (20 PFU per ml) when AHA was added (Fig. 3). To ensure that the addition of the plasminogen inhibitor AHA had an effect on only HA cleavage, not viral entry or replication, we next included trypsin, along with AHA as a control, in our viral growth assay. As shown in Fig. 3, the addition of exogenous trypsin could overcome the inhibitory effect of AHA; the viruses grown in the presence of both trypsin and AHA could undergo multiple rounds of replication, and the virus titer was comparable to that of virus grown without AHA, indicating that the plasmin/plasminogen specifically cleaves WSN HA in the absence of trypsin. Overall, our results suggest that the serine protease plasminogen, or its active form plasmin, is involved in WSN HA cleavage in MDBK cells. As described previously (16), WSN virus can be cleaved efficiently by serum plasminogen in the culture medium. To rule out the possibility of having serum plasminogen from the culture medium in our viral infection experiment, we washed MDBK cells extensively with PBS and cultured the cells with medium containing only BSA. When we examined multicycle replication of WSN in CV-1 cells, we found that the virus cannot undergo multicycle replication (data not shown), indicating that the plasminogen/plasmin activity responsible for WSN HA cleavage in MDBK cells is not from contaminating serum plasminogen in the medium but, instead, is associated with an intrinsic property of MDBK cells.

The surface-expressed HAs of the WSN wild type or the Y328F mutant, but not the Y328S mutant virus, are partially cleaved in MDBK cells. The serine protease zymogen plasmin-

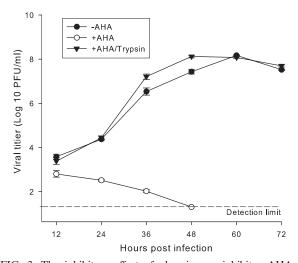


FIG. 3. The inhibitory effect of plasminogen inhibitor AHA on WSN multicycle growth in MDBK cells. MDBK cells were infected with WSN at an MOI of 0.001 for 72 h without trypsin or AHA (–AHA), without trypsin but with AHA (+AHA), or with both trypsin and AHA. The supernatants from infected cells were harvested every 12 h for plaque assay determination in MDBK cells with trypsin (0.5  $\mu g/ml$ ). The viral titer detection limit (20 PFU/ml) is shown as a dashed line. Error bars represent the standard error of the mean for three independent experiments.

ogen is often secreted from cells but can be cell surface associated based on interactions with surface-expressed receptors (44). If plasminogen/plasmin is indeed the protease responsible for WSN HA cleavage, we should expect to see the cleaved HA expressed in MDBK cells. To test whether this is the case, we examined the properties of surface-expressed HAs from virus-infected cells. MDBK cells were infected with either wildtype WSN or the Y328S or Y328F mutant virus at an MOI of 1 for 8 h in medium containing 0.2% BSA, with or without the addition of trypsin. HA1 (the cleaved product of HA0) could be detected by Western blotting at both the wild-type- and Y328F mutant virus-infected MDBK cell surface, even in the absence of trypsin in the infection medium, although the cleaved HA1 represented only a small fraction of total HA compared to the much more efficient cleavage by trypsin (Fig. 4). In contrast, the surface-expressed HA from Y328S-infected MDBK cells was essentially in the uncleaved HA0 form. When the same experiment was performed in CV-1 cells, no cleaved HA could be detected from either the wild-type or the Y328F or Y328S mutant virus-infected cells, indicating that HA cleavage of the WSN wild type or Y328F at the cell surface is cell type dependent and could occur in MDBK cells but not in CV-1 cells. We also examined HA cleavage for virions released from virus-infected MDBK cells. Consistent with the result showing the properties of surface-expressed HA, the virions released from wild-type- or Y328F-infected MDBK cells contained partially cleaved HA, whereas the virions from Y328Sinfected cells remained uncleaved, based on Western blot analysis (Fig. 4). To examine whether the cleaved HA from the WSN wild type- and Y328F-infected MDBK cell surface is derived from plasminogen/plasmin-mediated proteolysis, we added the plasminogen/plasmin inhibitor AHA (5 mg/ml) in the viral infection assay and found that the cleaved form of HA

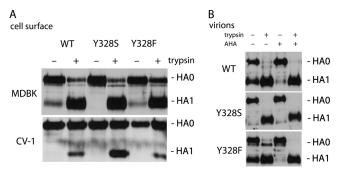


FIG. 4. WSN HA cleavage in virus-infected cells. (A) MDBK or CV-1 cells were infected with WSN wild type (WT) or with the mutant Y328S or Y328F at an MOI of 1 for 8 h or 16 h, respectively, in DMEM containing 0.2% BSA, and cell surface proteins were labeled with sulfo-NHS-SS-biotin, followed by immunoprecipitation with NeutrAvidin beads. For trypsin-treated samples, the infected cells were incubated with trypsin (1 µg/ml) for 20 min at 37°C before biotinylation. The surface-expressed HA was visualized by Western blotting using goat anti-A/PR8 polyclonal antibody. (B) MDBK cells were infected with the WSN wild type or with the mutant Y328S or Y328F virus at an MOI of 1 for 12 h in DMEM containing 0.2% BSA, with or without trypsin (1 μg/ml) and AHA (5 mg/ml). The supernatants of the infected cells were harvested, and cell debris was removed by centrifugation at 3,000 rpm for 5 min. Then the viruses in the harvested supernatants were collected by ultracentrifugation at 42,000 rpm for 2 h, and the viral pellets were resuspended in SDS-PAGE loading buffer, followed by Western blotting with goat anti-HA antibody.

could no longer be detected (Fig. 4). These results demonstrate that WSN HA can be cleaved at the MDBK cell surface before the virus is internalized into endosomal compartments and that cleavage is mediated by plasminogen/plasmin specifically associated with MDBK cells. In contrast, the failure of the Y328S virus to be cleaved at the cell surface results in abolished multicycle replication in MDBK cells in the absence of exogenous trypsin. In our study, we consistently observed a higher fraction of cleaved HA in the released WSN virions than of the cell surface-associated form (data not shown), likely due to the fact that newly synthesized HA is continually transported to cell surface but is not yet cleaved.

Plasminogen/plasmin preferentially cleaves HA with Y or F at the P2 position. As one of the most important serine proteases in the body, the substrate specificity for plasmin has been studied extensively (3). Although it was originally regarded to have substrate specificity similar to that of trypsin (which cleaves after either a lysine or arginine residue, with no preference for the P2 or P3 position), more recent studies using random six-amino-acid peptides displayed on phage surfaces or using positional scanning synthetic combinatorial libraries show that plasmin not only requires lysine or arginine at the P1 position but also has strong preference for the bulky aromatic residues tyrosine and phenylalanine at the P2 position (1, 17). The plasmin cleavage efficiency for the peptides, which differ in the P2 and P3 positions but have either lysine or arginine at P1 position, can vary up to 700,000-fold (17). To determine whether HA molecules with a variation at the P2 position could be differentially cleaved by plasmin, we performed the plasmin-mediated HA cleavage assay using our wild-type and mutant HAs expressed in 293 T cells in a time course experiment. Cleavage efficiency was calculated by the percentage of cleaved HA1 versus total HA based on Western blotting (Fig. 5). Through the time points we tested (10, 20, 30, 40, and 50 min), the plasmin-mediated cleavage efficiency of wild-type WSN HA (with a tyrosine at the P2 position, Y328) was very similar to that of Y328F HA but was at least 3-fold better than that of Y328S HA. After 50 min of incubation with plasmin (105 mU/ml), approximately 40% of wild-type and Y328F HA0 could be cleaved into HA1, in contrast to cleavage of only about 11% of the Y328S mutant HA. Our HA cleavage assay using exogenous plasmin therefore indicates that HA with a bulky aromatic residue at the P2 position of the cleavage site (i.e., tyrosine or phenylalanine) represents a more optimal substrate for plasmin and can partially explain why the HA of A/WSN/33 viruses, but not most other H1 HAs, can be cleaved endogenously in MDBK cells-based on a substitution of the conserved serine residue at the P2 position.

**Spread of wild-type and mutant viruses in mouse brain and lung.** To test whether the enhanced plasmin-mediated HA cleavability of WSN seen *in vitro* is also manifested *in vivo*, we evaluated infection and spread of both wild-type and mutant

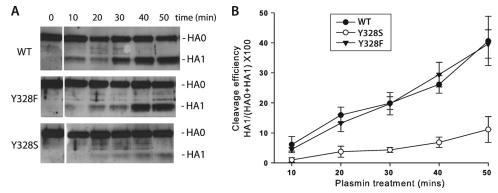


FIG. 5. Plasmin-mediated cleavage of WSN HA expressed in 293 T cells. (A) 293 T cells were transfected with either WSN HA wild type or with the mutant Y328S or Y328F for 24 h before being treated with plasmin for 0 to 50 min at 37°C. The surface proteins of the treated cells were then labeled with biotin, followed by NeutrAvidin bead immunoprecipitation. The surface-expressed HA was analyzed by Western blotting with anti-HA antibody. (B) The dose-dependent plasmin-mediated HA cleavage efficiency is expressed according to the formula HA1/(HA1 + HA0) ×100, with the data plotted in Sigma Plot, version 9.0.

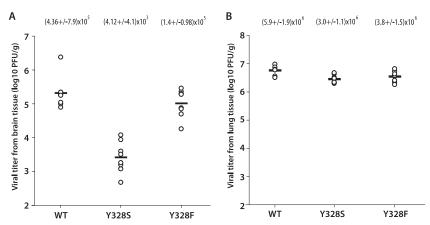


FIG. 6. Replication of WSN wild type and mutants in mouse brain and lung. Recombinant WSN wild-type virus or the Y328S or Y328F mutant was injected intracerebrally at  $1 \times 10^3$  PFU (A) or intranasally at  $2 \times 10^5$  PFU (B) into 4-week-old mice (eight per group). Viral titers in brain tissue after intracerebral infection or in lung tissue after intranasal infection at 3 days postinoculation were determined by plaque assay in MDBK cells with trypsin (0.5  $\mu$ g/ml). Circles represent the data from individual mice, and the mean viral titer for each group is shown with a solid bar.

viruses in both neurotropic and pneumotropic mouse models. To examine viral multicycle replication in the brain, eight 4-week-old BALB/c mice were injected intracranially with 1,000 PFU of either the recombinant WSN wild type or Y328F or Y328S mutant virus, and brain tissue was collected 3 days postinfection. Viral titers in the brain tissue were then determined by plaque assay in MDBK cells in the presence of trypsin. As shown in Fig. 6A, the viral titers from WSN wildtype- and Y328F mutant-inoculated mouse brain were not significantly different (P = 0.52), with a mean value of 4.36  $\times$  $10^5$  and  $1.40 \times 10^5$  PFU per gram of brain tissue, respectively. In contrast, the viral titer from the Y328S mutant-inoculated group was significantly different from that of the wild type (P =0. 0002), with virus titers over 2 logs lower  $(4.13 \times 10^3 \text{ PFU per})$ gram) than the titer of the wild type. These data indicate that the Y328S mutant virus loses the ability to replicate efficiently in mouse brain and becomes less neurovirulent.

We next assessed the pathogenicity of WSN HA wild-type and mutant viruses in lung tissue. Eight 4-week-old mice per group were inoculated intranasally with  $2 \times 10^5$  PFU of recombinant WSN wild-type or Y328F or Y328S mutant virus, and lung tissues were collected 3 days postinfection to determine virus titer. The mean titers of the viruses recovered from each group were  $5.90 \times 10^6$  PFU per gram of lung tissue for WSN wild type,  $3.80 \times 10^6$  PFU per gram of lung tissue for Y328F, and  $3.02 \times 10^6$  PFU per gram of lung tissue for Y328S (Fig. 6B). While the titer of Y328S following intranasal inoculation did show a statistically significant difference compared to the titer of the wild-type virus (P = 0.0044), the resultant virus titer showed a difference of less than 1 log unit. The titer of Y328F following intranasal inoculation was not significantly different from that of the wild type (P = 0.058). We therefore conclude that the Y328S mutant is significantly attenuated in its ability to spread in mouse brain but is less attenuated for viral spread in the lung. The Y328F mutant is very similar to wild-type WSN in viral pathogenicity in both brain and lung.

### DISCUSSION

In this study, we present evidence that variation in the P2 position of the HA cleavage site from an H1N1 influenza virus is directly linked to viral pathogenicity. In particular, we show that the high neurovirulence of the A/WSN/33 virus in mice is partially due to a tyrosine substitution at the P2 position, compared to the conserved serine residue shared by almost all other H1 HAs. *In vitro*, H1 HA with tyrosine or phenylalanine at the P2 position, which represents an optimized substrate, can be cleaved more efficiently by the serine protease plasmin than an HA with a serine residue at this position. Having an optimized substrate sequence for plasmin cleavage is crucial for in vitro viral multicycle replication in serum-free MDBK cells and also for in vivo replication in mouse brain. In both cases, the available plasmin or plasminogen might be very limited compared to the plasminogen normally present in cell culture serum, explaining why HA cleavage and virus propagation under serum-free conditions is greatly affected by the sequence at the cleavage site.

We suggest that A/WSN/33 and A/NWS/33 viruses, selected by extensive passaging of parental A/WS/33 virus in mouse brain, have not only accumulated mutations in the NA gene to recruit plasminogen and locally enhance plasminogen availability but have also selected mutations in HA to facilitate plasmin-mediated cleavage by the cell-associated plasminogen. Our results indicate that a bulky aromatic residue at the P2 position of WSN HA contributes greatly to viral neurovirulence, most likely because it allows efficient HA cleavage by plasminogen residing in brain tissue (4, 29). In contrast, the proteases responsible for WSN HA cleavage in respiratory organs might have no preference for specific residues at the P2 position, as lung tissue expresses a variety of trypsin-like proteases (2, 6, 9) that are less likely to have a preference for specific residues at P2 position. While trypsin-like enzymes have been shown to act as candidate proteases for HA cleavage in the brain (19, 23), our data indicate that plasmin/plasminogen is a major discriminating factor that allows A/WSN/33 and A/NWS/33 viruses to spread efficiently in the brain.

As host proteases are required for influenza virus replication *in vivo*, the exogenous addition of trypsin in the cell culture medium is often necessary for propagating influenza viruses *in vitro*. However, several cell lines, for example, human HepG2 cells and certain MDCK cells, have been shown to support influenza virus replication in the absence of trypsin because these cell lines can either express HA-activating proteases or recruit proteases from tissue culture serum (31, 32). In contrast to HepG2 and MDCK cells, the trypsin-independent replication of influenza virus in MDBK cells is unusual because support for viral replication is restricted to A/WSN/33 and is also WSN NA dependent. This suggests not only that WSN NA can facilitate the recruitment of host cell proteases but also that WSN HA may consist of special structural elements to facilitate its cleavage.

In our study, we demonstrate that WSN HA cleavage in MDBK cells is mediated by the serine protease plasmin and that a tyrosine or phenylalanine residue at the P2 position of the HA cleavage site is critical for plasmin-mediated cleavage. Because plasminogen/plasmin are critical proteases involved in many biological functions, including fibrinolysis and wound healing as well as tissue homeostasis, the production and activation of plasminogen/plasmin are tightly regulated (44). In the host, zymogen (plasminogen) is synthesized mainly by liver cells, and activation from plasminogen to the active form of plasmin depends mainly on its cleavage by plasminogen activators, which include both tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) (34, 35). Although it has been shown previously that WSN NA can recruit plasminogen to infected cells, it is not yet clear what factor activates the recruited plasminogen to plasmin, either in vitro or in vivo. To date, we cannot define the source for the plasmin associated with the MDBK cells in our study. However, we speculate either that MDBK cells can secrete plasminogen or that MDBK cells express higher levels of plasminogen activators at the cell surface, which can facilitate the production of plasmin from residual plasminogen even after extensive washing of cells with PBS. It has been reported that some nonhepatic cells express higher levels of mRNAs corresponding to plasminogen (48); however, we have failed to detect plasminogen at the MDBK cell surface or in conditioned culture medium by Western blotting, indicating that the level of the expressed plasminogen from MDBK cells is very low. Regardless of the source of plasminogen, the presence of WSN NA can further enhance the concentration of plasminogen locally around WSN HA by recruiting plasminogen by means of the viral NA, consistent with our hypothesis that WSN HA, which represents an optimized plasmin substrate, can be efficiently cleaved and activated.

A trypsin-independent phenotype has also been described for the 1918 pandemic H1N1 influenza virus (8, 43), and similarities in HA cleavage between 1918 influenza virus and WSN have been suggested. However, the 1918 virus NA has been shown to be unable to recruit plasminogen (8), and also the 1918 virus HA has no bulky aromatic residue at position P2 to allow it to be cleaved more efficiently by plasminogen (Fig. 1). The mechanisms underlying HA cleavage therefore appear to differ between A/WSN/33 and 1918 influenza viruses. It may be

possible that the 1918 virus NA recruits a different protease and that the recruited protease specifically recognizes the HA of the 1918 virus or that the enzymatic activity associated with 1918 virus NA allows HA to have different glycan modifications—which may subsequently enhance the access of the proteases to HA. In the future, a similar approach described in our study might be applied to the investigation of the molecular mechanism of 1918 influenza virus trypsin-independent viral replication and may help us understand the high virulence of 1918 influenza virus.

While A/WSN/33 and A/NWS/33 are laboratory-adapted viruses, a functional role for activation of influenza virus by plasmin/plasminogen is also likely in clinical situations. In addition to the activity of endogenous tissue plasminogen, coinfecting bacteria present in the respiratory tract, such as Staphylococcus and Streptococcus spp., produce plasminogen activators that can be used to cleave HA (5, 28, 36, 42). It has also been reported that plasmin-mediated activation may not be a unique feature of the HA of A/WSN/33, as other influenza viruses such as A/Udorn/72 (H3), may recruit plasminogen to the surface of the virus particle via annexin II on the viral envelope (24). In the case of influenza A/WSN/33 virus, a maximal effect of the plasmin/plasminogen system has likely resulted from multiple genomic mutations in the selection of the virus from A/WS/33 (with mutations in the NA as well as in HA distal to the cleavage site and potentially in other gene segments). The data presented here suggest that the acquisition of a bulky hydrophobic residue (such a tyrosine or phenylalanine) at the P2 position of the HA cleavage site may have profound effects on the pathogenic potential of any influenza virus.

Although influenza virus is mainly pneumotropic in humans, it can occasionally cause encephalopathy or encephalitis (11, 14). Influenza viral antigen and genome have been detected in cerebrospinal fluid and brain tissue in human lethal cases, and influenza virus has also been speculated to contribute to the higher occurrence of central nervous system disorders in humans during and following pandemics (13, 26, 41). To date, the proteases that might be responsible for influenza virus HA cleavage in the human brain are poorly understood. Although it has been shown that trypsin I isolated from rat brain and murine mini-plasmin accumulated in the cerebral capillaries were capable of cleaving HA (23, 47), their role in influenza virus neuropathogenesis has not been established in vivo in humans. In the future, it will be of great interest to further define the role of plasmin and the activation of plasminogen in influenza virus neurovirulence in clinical situations. It will also be of critical importance to monitor surveillance data of influenza virus isolates for changes in the P2 position of the HA cleavage site (and in other positions) that may allow enhanced virus activation by proteases such as plasminogen/plasmin and increased virulence and spread in the host.

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